CHEMORECEPTION IN THE MUD SNAIL, NASSARIUS OBSOLETUS. I. PROPERTIES OF STIMULATORY SUBSTANCES EXTRACTED FROM SHRIMP[1]

WILLIAM E. S. CARR[2]

Department of Zoology, Duke University, Durham, North Carolina

Chemoreception is believed to play a role in many aspects of gastropod behavior (Kohn, 1961). Studies have suggested that chemoreceptive mediation is involved with such diverse phenomena as carrion location by scavengers (Copeland, 1918), metamorphosis (Scheltema, 1961), plant recognition by herbivores (Frings and Frings, 1965), predator avoidance (Bullock, 1953), prey recognition by predators (Blake, 1960), sexual differentiation (Coe, 1953), and others. The compilation of a sizable literature on the ecological significance of chemoreception to aquatic gastropods and aquatic invertebrates in general (for review, see Hodgson, 1955) is contrasted with the paucity of available information on the molecular aspects of such problems. A satisfactory understanding of aquatic chemoreceptive phenomena can be attained only after insight is gained into the molecular nature of the compounds which are involved.

Previous studies on chemoreception in marine gastropods have been limited in their scope by the fact that the animals selected for study and/or the techniques employed have denied investigators a chance to progress much beyond the "observation of response" stage of investigation. However, in the present study the mud snail, *Nassarius obsoletus,* is shown to be an extremely suitable animal for studies of chemoreception. Dimon (1905) noted that this marine gastropod possessed a chemical sense and could detect the "odor or taste" of substances which diffused from dead animals. Submerged individuals were observed by Copeland (1918) to respond to extracts of fish by extending their proboscides. Copeland referred to this response as the proboscis reaction. Prior to the present study no attempt has been made to identify the substances which are stimulatory to this snail.

The report which follows has a twofold purpose: (1) to describe a procedure for studying a chemically mediated response in *N. obsoletus;* and (2) to provide the results of experiments which characterized the response-inducing substances extracted from shrimp.

[1] This paper is based on a portion of a dissertation submitted to the Graduate School of Arts and Sciences of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology. These studies were conducted at the Duke University Marine Laboratory, Beaufort, N. C., and were supported by a N.I.H. Physiology Training Grant and by a Bureau of Commercial Fisheries Graduate Education Grant. The author is grateful to Dr. K. M. Wilbur, Department of Zoology, Duke University, for providing frequent counsel during the course of this research.

[2] Present address: Department of Zoology, University of Florida, Gainesville, Florida 32601.

Methods

Maintenance of animals

Specimens of *Nassarius obsoletus* were collected near the Duke University Marine Laboratory, Beaufort, N. C. Prior to use, freshly collected snails were kept under running sea water in the laboratory for 12 to 14 hours for washing and egestion of intestinal contents. For further washing and a period of adjustment to room temperature, groups of approximately 200 snails were transferred to 8-liter glass aquaria filled with freshly pumped and aerated sea water. After the water reached room temperature (21–26° C.) it was replaced several times over a 24-hour period with water at the same temperature. Groups of 45 snails each were then transferred to 8-liter aquaria, each containing approximately 4 liters of filtered and continuously aerated sea water which was changed after three days. No tests were performed with snails which had been in the laboratory less than 48 hours. Snails were used no longer than 8 days after capture. Snails were seldom used for more than one test and never less than 24 hours after a previous test.

Snails with heavily eroded or encrusted shells or with obvious physical imperfections were not used. Between mid-December and early May only males were used in order to avoid variables resulting from copulation and capsule deposition in mixed populations. At other times mixed populations were used.

In order to keep organic contamination to a minimum, sea water for maintaining and testing washed snails was obtained from Beaufort Inlet at high tide. Water was collected in glass carboys, filtered, stored at 4° C., and warmed to room temperature as needed. The sea water in which snails were tested was always collected at the same time as the water in which these same snails were maintained. The salinity range was 29–35‰.

Bioassay procedures

Bioassays were performed in an illuminated observation cubicle which was provided with a mirror to permit observation of the behavior of snails moving away from the observer (Fig. 1). Solutions were bioassayed in 10-cm. petri dishes. A solution volume of 40 ml., sufficient to cover the siphonal canal, was used. Bioassays were standardized as follows:

(1) Ten snails (comprising one test group) were transferred from an aquarium into a fingerbowl of sea water dipped from the aquarium. The snails were placed in the observation cubicle for 10–15 minutes prior to beginning the bioassays.

(2) Individuals were transferred with stainless steel forceps into the solution which was to be bioassayed. The snails were tested one at a time. A one-minute testing period was allowed for displaying the proboscis search reaction (description in Results).

(3) A single solution was used for one test group. Forceps used in the transfer of snails were rinsed thoroughly in distilled water after each transfer. Water in the containers used for rinsing snails or forceps (see Fig. 1) was changed after each series of bioassays involving a test group.

Preparation of shrimp extracts

The shrimp (*Penaeus duorarum*) used as the source of tissue for the studies of response-inducing substances were transported to the laboratory alive, placed into

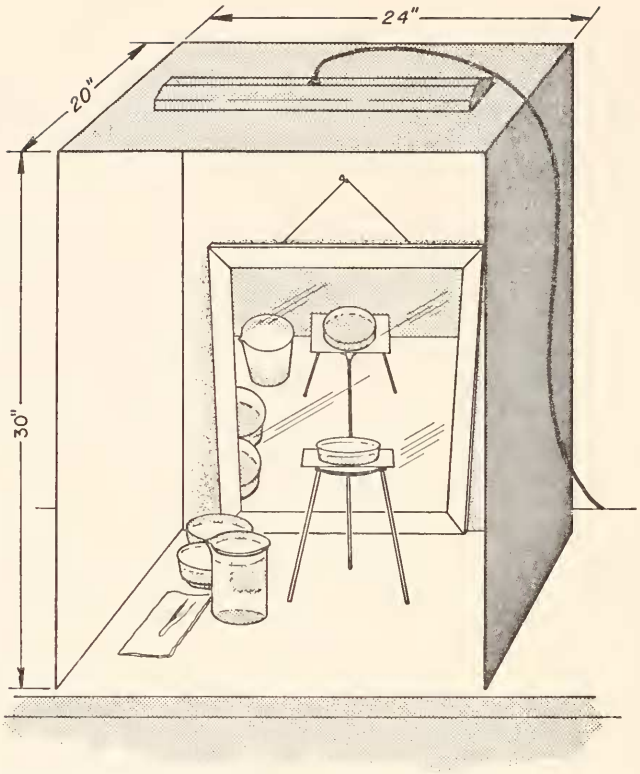

FIGURE 1. The observation cubicle. The cubicle was constructed from half-inch plywood and had the following dimensions: height = 30 in.; width = 24 in., depth = 20 in. A slot cut from the top provided for a framed 15-watt, Cool White General Electric fluorescent light. The mirror (39 × 49 cm.) hanging diagonally in the rear of the cubicle permitted the observation of the behavior of snails moving away from the observer. The following items were located within the cubicle: (1) forceps used for transferring snails; (2) ring stand to support test solution; (3) fingerbowl of water to rinse snails after they were used; (4) fingerbowl of sea water to hold snails after they were used; and (5) beaker of distilled water for rinsing forceps.

plastic bags, and stored in a deep freeze. For all extracts the abdomen was used without appendages and exoskeleton. Extracts were prepared in two ways:

(1) Aqueous extracts (10:1, v/w) were prepared by homogenization of shrimp for 5 minutes in a Waring Blendor. The homogenates were filtered with suction through Whatman No. 5 paper, refiltered through a Millipore filter (0.45-μ pore size), and kept in ice.

(2) Chloroform:methanol (2:1) extracts were prepared according to the procedure of Folch, Lees and Sloane Stanley (1957). After addition of 0.2 volume of water to the filtered homogenate, the preparation was stirred for several minutes and phase separation was accelerated by centrifugation at 400 g for 20 minutes. The upper aqueous phase was removed, evaporated to dryness in an oven at 50° C., suspended in water (1 ml./1 g. initial shrimp weight), filtered through a Millipore

filter, and kept in ice. Upper phase material was used extensively and will be designated shrimp extract. Lower phase material was not used except when specified.

Shrimp was selected as the source of tissue for these studies because of its availability and because only a minimum amount of manipulation is necessary to prepare it for extraction.

Procedures for studying the properties of stimulatory substances extracted from shrimp

The following properties of the stimulatory substances extracted from shrimp were studied: stability to heat, solubility, volatility, dialyzability, and stability to acid and ammonia treatment. The molecular-charge properties of the stimulatory substances were studied by means of a series of fractionations of extracts on ion exchange columns. In each experiment, a portion of an extract was treated (or fractionated) in the manner described below. Tests were carried out which compared the response-inducing capacity of the treated (or fractionated) extract with the response-inducing capacity of the untreated (or total) extract.

Whenever necessary, solutions were evaporated to dryness by rotary evaporation *in vacuo*. All preparations were kept in ice and tested as soon after preparation as possible.

Heat treatment. Sealed aliquots of aqueous shrimp extract were suspended in a boiling water bath for periods of 1 and 12 hours. The preparations were filtered and tested.

Solubility studies. Prior to deciding upon an extraction procedure to be used for further studies, it was necessary to get an approximation of the relative solubility of the stimulatory substances in a series of solvents of differing polarity. Ten-ml. aliquots of aqueous shrimp extract were evaporated to dryness and to each residue were added 10 ml. of one of the following solvents: water, methanol, ethanol, chloroform : methanol (2 : 1), chloroform : methanol : water (3 : 48 : 47), acetone, ether. The containers were stoppered, placed on a shaker for 30 minutes, filtered, and evaporated to dryness. Each residue was dissolved in 10 ml. of water and tested.

Ultrafiltration. An aliquot of shrimp extract was ultrafiltered at 0–1° C. through 6-mm. Visking tubing according to the procedure of Smith (1960, pp. 60–62). The ultrafiltrate was tested.

Acid hydrolysis. A 1-ml. aliquot of shrimp extract was hydrolyzed in 6 N HCl (20 hours, 100° C.). The hydrolysate was evaporated to dryness and the residue was dissolved in 1 ml. of water, neutralized with NaOH, and tested.

Ion exchange separations. The neutral compounds in shrimp extract were collected by passing a 2-ml. portion of extract through a 1.2 × 12-cm. column of Bio-Rad AG 501-X8 (mixed bed resin, Analytical Grade, 20–50 mesh, Calbiochem). Ten bed volumes of water effluent were collected at a flow rate of 0.5 ml. per minute. The effluent containing neutral compounds was evaporated to dryness and the residue was dissolved in 2 ml. of water for testing.

The neutral, acidic, amphoteric, and weakly basic compounds in shrimp extract were separated from the strongly basic compounds by passing a 3-ml. portion of

extract through a 1 × 15 cm. column of Rexyn 102 (Chromatographic Grade, 200 mesh, Fisher). The procedure of Awapara *et al.* (1960) for the separation of amino acids from amines was used with the following modifications: (1) 10 bed volumes of water effluent were collected; (2) elution was carried out with 10 bed volumes of 1 N HCl. The effluent (containing neutral, acidic, amphoteric, and weakly basic substances) and the eluate (containing strongly basic substances) were collected at a flow rate of 0.5 ml. per minute. Each was evaporated to dryness and the residue was dissolved in 3 ml. of water for testing.

A 1 × 11 cm. column of Dowex 50W-X8 (Analyzed Reagent, 200–400 mesh, Baker) was used according to the procedure of Smith (1960, p. 59) to separate the components of shrimp extract into 3 fractions: (1) a water effluent containing primarily neutral and acidic compounds; (2) a 2 N-NH_3 eluate containing amphoteric and weakly basic compounds; and (3) a 10 N-NH_3 eluate containing strongly basic compounds. One ml. of extract was added to the column and 10 bed volumes of each of the above fractions were collected at a flow rate of 0.5 ml. per minute. Each fraction was evaporated to dryness and the residues were dissolved in one ml. of water for testing. In a separate experiment, 2.5 ml. of extract were added to a 1 × 15 cm. column of Dowex 50W-X8. Ten bed volumes each of water effluent and 2N-NH_3 eluate were collected, evaporated to dryness, and the residues were dissolved in 2.5 ml. of water for testing.

Statistical treatment of data

Many of the data which follow were analyzed by Potency Probit Analysis employing the computer program of Daum and Givens (1963) for quantal (all-or-none response) bioassays. This program employs the maximum likelihood procedure of Finney (1962) and involves a regression analysis of the $\log_{10}$-dose *versus* probit response for a series of up to 5 simultaneous sets of data. The potency (ratio of equally effective doses) and its 95% confidence limits are computed for an "unknown"(s) with respect to a "standard." This analysis also provides calculation of an ED_{30} (effective dose for 30% of tested animals), ED_{50}, ED_{70}, and ED_{90} for each set of data which comprises a significant linear regression; these calculations permit the plotting of computed regression lines whenever it is desirable to present such material graphically. The computer program was provided by Mr. Kenneth Fischler, Biometrician, U. S. Fish and Wildlife Laboratory, Beaufort, N. C. The Potency Probit Analysis was carried out in the Duke University Digital Computing Laboratory.

Results and Discussion

In the field *Nassarius obsoletus* quickly detected substances which leached from freshly killed crabs, fish, oysters and other animals. Submerged snails which were below the tide mark, where slight currents were sufficient to transport diffusing substances, were observed to move upcurrent directly to such material from distances of two or three feet. Such movements were accompanied by a horizontal waving of the siphon. Once a snail was within a few centimeters of fresh tissue, the food search was climaxed by a series of short and/or long extensions of the previously unexposed proboscis (Fig. 2). The initial contact of a snail with food

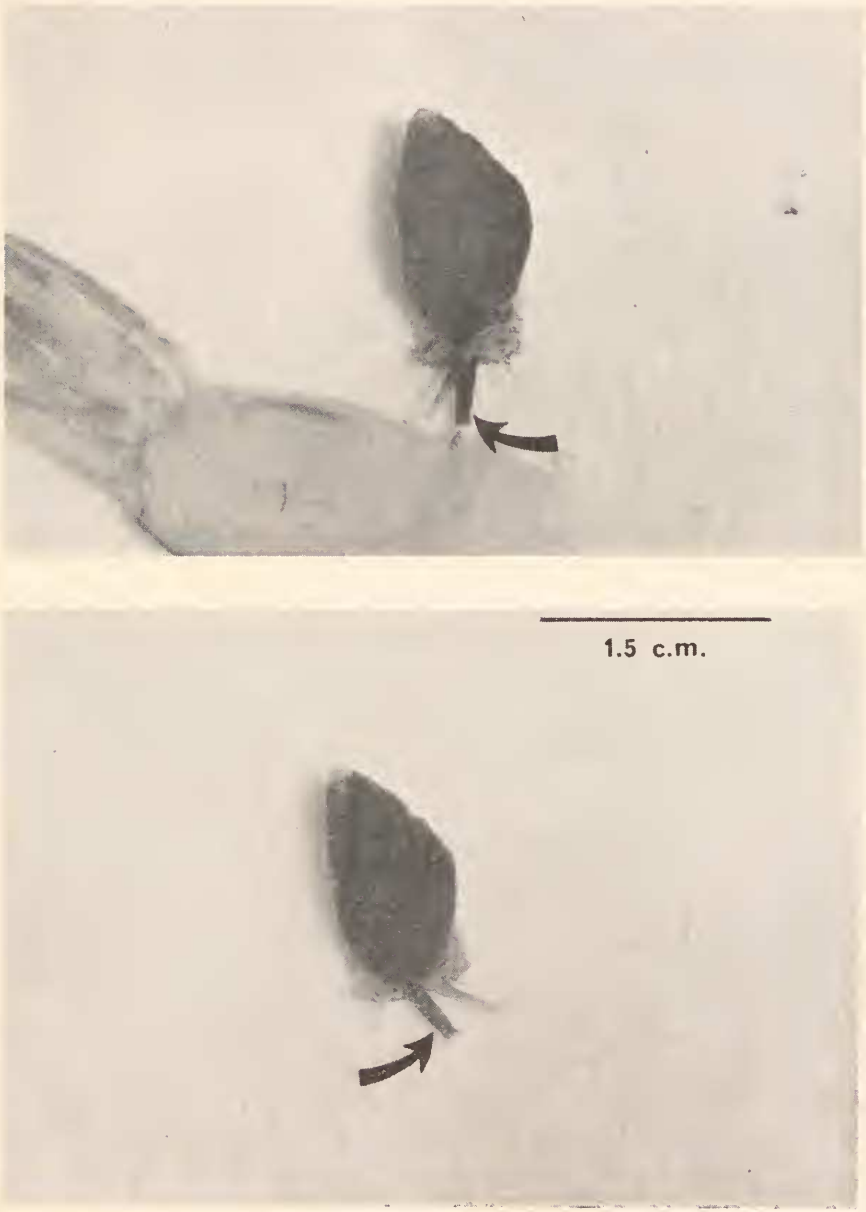

FIGURE 2. *N. obsoletus* responding to components released from fresh shrimp. The snail in upper photograph is submerged in sea water containing a portion of a shrimp (lower center). This snail is extending its proboscis (indicated by arrow) in response to substances diffusing from the shrimp. The snail in lower photograph is submerged in sea water which contained a portion of a shrimp prior to the introduction of the snail. This snail is extending its proboscis (indicated by arrow) in response to substances which were released from the shrimp.

was usually made by the proboscis; however, extensions of the proboscis (hereafter referred to as the proboscis search reaction [PSR]) always began before the snail contacted the food. In tidal pools two to four feet in diameter, the introduction of freshly killed oysters, fish or crabs resulted in accelerated locomotion and siphon waving as well as emergence from the mud of previously buried snails. Without the directional stimulus or gradient provided by a current, the movement of snails was frequently unrelated to the position of the food. Nevertheless, individuals which moved within a few centimeters of the food would begin the characteristic PSR and quickly locate the stimulus source. Early laboratory experiments revealed that the actual presence of food in sea water was not required to induce the PSR. The PSR was stimulated by the presence of sufficient concentrations of certain tissue substances which leached out after injury or death.

The distinctive feature of the PSR is a series of short (just beyond the anterior end of the siphonal canal) and/or long (siphon length) extensions of the proboscis. The reaction occurs within a few seconds. Preliminary observations on several hundred snails tested individually suggested that the response of an individual to a control or test solution during a 1-minute test period could be graded as follows:

0–3 extensions of the proboscis = No response
4 or more extensions of the proboscis = Satisfactory response.

These response criteria were used throughout. The initial expansion of the foot by a snail placed in a control or a test solution was sometimes accompanied by a short extension of the proboscis; also contact of a snail with the side of a testing dish sometimes resulted in one or two short extensions. In order to exclude such responses the grading system was established. The terms, stimulation, stimulatory solution, response, response-inducing solution, are references to the PSR or to a solution which induced the PSR.

Studies of the properties of stimulatory substances extracted from shrimp

Preliminary experiments revealed that the addition of small amounts of aqueous shrimp extracts to sea water yielded very stimulatory solutions. The following experiments were carried out to provide information on the properties of the stimulatory substances. In each of these experiments, tests of the untreated (or total) extract were conducted together with tests of the treated (or fractionated)

TABLE I

Responses of N. obsoletus to untreated and heat-treated shrimp extract

Extract concentration tested (μl./40 ml.)	Untreated extract		1 hr. heat-trtd. extract		12 hr. heat-trtd. extract	
	(1) No. snails tested	(2) % Response	(3) No. snails tested	(4) % Response	(5) No. snails tested	(6) % Response
0	10	10				
5	20	40	20	45	20	40
10	20	70	10	60	10	80
20	10	70	10	100	10	70

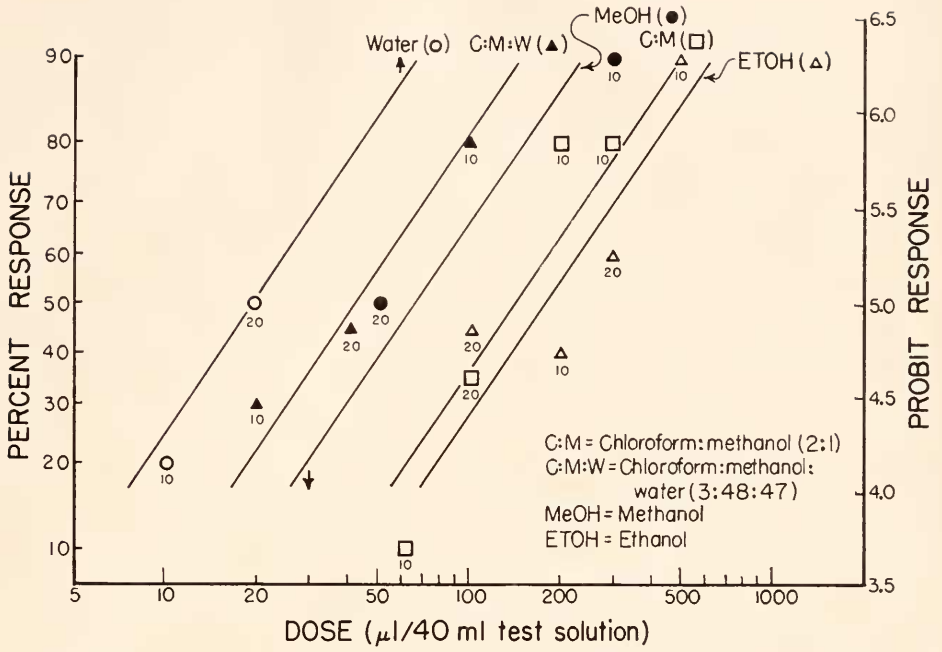

FIGURE 3. Responses of *N. obsoletus* to shrimp components soluble in water and organic solvents. The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. Linear regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. The upright arrow over the regression line for water-soluble substances indicates that a 100% response was obtained with the largest dose tested. The inverted arrow beneath the regression line for methanol-soluble substances indicates that no response (0%) was obtained with the smallest dose tested. The potencies (and 95% confidence limits) of the indicated preparations were computed with respect to the potency of the preparation containing water soluble substances:

	Upper lim.	0.867		Upper lim.	0.257
C:M:W	Potency	0.469	C:M	Potency	0.144
	Lower lim.	0.253		Lower lim.	0.079
	Upper lim.	0.577		Upper lim.	0.202
MeOH	Potency	0.307	ETOH	Potency	0.113
	Lower lim.	0.157		Lower lim.	0.065

extract. This measure insured that all groups of snails used in comparative tests were in the same physiological condition.

Effect of heat treatment. The results of bioassays of heat-treated and untreated shrimp extract are given in Table I. Heat treatment had no detectable effect upon the stimulatory capacity of the extract. This conclusion was based upon a comparison of the response percentages in columns 2, 4, and 6 of the Table.

Solubility studies. Figure 3 incorporates the experimental results of the solubility studies and the dosage-response regression lines obtained by a Potency Probit Analysis (see Materials). The figure shows the relative solubility of the stimulatory substances (from shrimp) in water, methanol, ethanol, chloroform:methanol, and chloroform:methanol:water. The stimulatory substances were somewhat

more soluble in water than in the other solvents. However, with respect to solvents other than water, only the data from tests of substances soluble in chloroform: methanol:water and ethanol are significantly different. The figure emphasizes the existence of a relationship between the concentration of a stimulant and the percentage of snails responding to it. This relationship implied that different individuals possessed different thresholds and that the procedure of recording response as a function of dose over a range of concentrations provided a means of comparing solutions in terms of their stimulatory capacities. This procedure was used in the studies which follow. One of the shortcomings in using small numbers of animals is exemplified in the figure by the aberrant point obtained with ethanol-soluble material; a 40% response (10 animals tested) was obtained with a dose of 200 μl./40 ml. while a 45% response (20 animals tested) was obtained with a lesser dose of 100 μl./40 ml. Deviations such as this were compensated for by bioassaying solutions over a range of concentrations (minimum of three unless otherwise stated) and basing most evaluations upon a Potency Probit Analysis.

The stimulatory substances were only very slightly soluble in acetone and ether; data from these tests were not included in Figure 3. At concentrations of 5000 μl./40 ml., the substances soluble in the non-polar solvents (acetone and ether) induced but 2 responses (20%) each from single groups of snails. Since the water-soluble substances induced a 50% response at a concentration of only 20 μl./40 ml., the limited effectiveness of the substances soluble in the non-polar solvents implied that lipoidal and long-chain aliphatic substances were not the major response-inducers.

The extraction procedure of Folch *et al.* (1957) incorporated several features which made it a suitable technique for the acquisition of substances for further studies. After initial homogenization in chloroform:methanol (2:1), followed by the addition of 0.2 volume of water, a biphasic system results: an upper aqueous phase (chloroform:methanol:water, 3:48:47) and a lower lipoidal phase (chloroform:methanol:water, 86:14:1). The extract is essentially protein-free and after phase separation the upper phase is nearly lipid-free. The previous experiment showed that the solubility of the stimulatory substances in chloroform:methanol: water (3:48:47) was at least as great as the solubility of these substances in methanol and ethanol (see Fig. 3). An experiment was carried out to observe the distribution of the stimulatory substances in the biphasic system described above. The results revealed that the substances which were response-inducing at low concentrations were confined to the upper aqueous phase. The substances in the lipoidal phase were effective only at concentrations which were approximately tenfold greater. Further references to shrimp extracts signify the utilization of the upper phases from extracts prepared according to the procedure of Folch *et al.*

Tests of volatile components. The fact that previous extracts were evaporated to dryness prior to use suggested that volatile components were not the ones stimulating the PSR. Nevertheless the findings of Brown (1961) and Kleerekoper and Mogensen (1963), concerning the chemoreceptive importance of volatile amines to certain aquatic animals, prompted experiments to collect and bioassay volatile components. However, the response-inducers extracted from shrimp were non-volatile substances. Volatile amines collected from shrimp by steam distillation were not response-inducing. Equally ineffective were volatile substances which

TABLE II

Responses of N. obsoletus to total and ultrafiltered shrimp extract

Concentration tested (μl./40 ml.)	Untreated extract		Ultrafiltrate of extract	
	No. snails tested	% Response	No. snails tested	% Response
0	10	0		
10	10	30	10	30
20	20	45	20	55
40	10	70	10	80

Potency* (and 95% confid. lims.) of ultrafiltrate

Upper limit	3.95
Potency	1.27
Lower limit	0.60

* The potency of the ultrafiltrate was computed with respect to the potency of the untreated extract.

were drawn from shrimp by negative pressure and trapped in cold sea water for testing.

Effect of ultrafiltration. The results of bioassays of shrimp extract and an ultrafiltrate of the same are given in Table II. The ultrafiltrate was as effective as the total extract. The results indicated that the principal response-inducers were substances of low molecular weight.

Effects of acid and alkaline hydrolysis. Shrimp extracts contained an acid-labile component(s) which contributed to the stimulatory capacity. Extract hydrolyzed in 6 N HCl was markedly less effective than untreated extract (Table III). Another similar experiment gave similar results. In a separate experiment, portions of shrimp extract were treated with 2 N HCl or 2 N NH_3 (1 hour at

TABLE III

Responses of N. obsoletus to untreated and acid-hydrolyzed shrimp extract

Concentration tested (μl./40 ml.)	Untreated extract		Acid-hydrolyzed extract	
	No. snails tested	% Response	No. snails tested	% Response
0	10	0	—	—
5	10	10	—	—
10	20	50	—	—
20	20	65	10	10
40	—	—	20	25
80	—	—	20	65

Potency* (and 95% confid. lims.) of acid-hydrolyzed extract

Upper limit	0.312
Potency	0.194
Lower limit	0.108

* The potency of the acid-hydrolyzed extract was computed with respect to the potency of the untreated extract.

100° C.). The acid treatment resulted again in a decrease in the stimulatory capacity, while treatment with NH_3 did not.

Effect of oxidation. Although no single experiment was designed to observe the effect of oxidation, other treatments (*i.e.*, evaporation of extract to dryness in an oven at 50° C. and retention of activity after 12 hours in boiling water bath) implied that readily oxidizable substances were not among the principal contributors to the stimulatory capacity of shrimp extracts.

Ion exchange separations. The neutral compounds in shrimp extract were collected by passing a portion of the extract through a column of mixed bed resin. Bioassays revealed that the neutral compounds were essentially non-stimulatory

TABLE IV

Responses of N. obsoletus to shrimp extract and to extract components collected in water effluent and acid eluate from a column of Rexyn 102

Material tested	Concentration tested (μl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
Total shrimp extract	0	10	0	
	2	20	35	
	5	20	55	
	10	20	60	
	20	20	75	
Water effluent from Rexyn 102 column	5	20	50	Upper lim. 3.45
	10	20	55	Potency 0.68
	20	20	65	Lower lim. 0.14
Acid eluate from Rexyn 102 column	20	10	0	
	40	10	0	
	100	10	0	
	250	10	10	
Water effluent plus acid eluate	5	10	50	
	10	10	60	

* Potency (and 95% confidence limits) of water effluent was computed with respect to potency of the total shrimp extract.

even at high concentrations (concentrations proportional to 250 and 500 μl. of extract per 40 ml.). The total extract itself induced a response of 55% at a concentration of only 5 μl./40 ml. These results verified those obtained from an earlier similar separation which was bioassayed over a smaller range of concentrations.

Shrimp extract was passed through a column of Rexyn 102 to separate the neutral, acidic, amphoteric, and weakly basic compounds from the strongly basic compounds. Results of the tests are given in Table IV. The compounds in the water effluent (neutral, acidic, amphoteric, and weakly basic compounds) possessed a stimulatory capacity comparable to that of the total extract. The acid eluate (containing strongly basic compounds) was very ineffective. Combinations of the effluent and eluate were not detectably more effective than the effluent alone. These

results verified those obtained from an earlier, similar separation which was bioassayed over a smaller range of concentrations.

The previous experiment suggested that the principal contributors to the response-inducing capacity of shrimp extracts were included with the neutral, acidic, amphoteric, and weakly basic compounds. Shrimp extract was passed through a column of Dowex 50W-X8 to separate the neutral and acidic compounds (collected in the water effluent) from the amphoteric and weakly basic compounds (collected in the 2 N-NH_3 eluate). Strongly basic compounds were collected by a terminal elution of the column with 10 N NH_3. Results of the tests are given in Table V. The components present in the 2 N-NH_3 eluate were markedly more

TABLE V

Responses of N. obsoletus to shrimp extract and to extract components present in water effluent, 2 N-NH_3 eluate, and 10 N-NH_3 eluate from a column of Dowex 50W-X8

Material tested	Concentration tested (μl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
Total shrimp extract	0	20	10	
	10	20	50	
	20	20	65	
	40	20	70	
Water effluent from Dowex 50 column	20	10	20	
	40	10	20	
	100	10	10	
2 N-NH_3 eluate from Dowex 50 column	10	20	30	Upper lim. 0.946
	20	30	43	Potency 0.341
	40	10	60	Lower lim. 0.011
	100	10	70	
10 N-NH_3 eluate from Dowex 50 column	20	10	0	
	100	20	15	

* Potency (and 95% confidence limits) of 2N-NH_3 eluate was computed with respect to potency of the total shrimp extract.

effective than the components present in either the water effluent or the 10 N-NH_3 eluate. However, components in the 2 N-NH_3 eluate were somewhat less effective than the total extract. The results were in accord with those obtained from an earlier, similar separation which was bioassayed over a smaller range of concentrations.

The finding that the extract components in the 2 N-NH_3 eluate from the Dowex 50 column were less effective than the total extract suggested that one of the other fractions collected from the column contained an important component(s). The Rexyn 102 separation suggested that strongly basic compounds did not contribute to the extract's stimulatory capacity (see Table IV). This made it seem likely that the water effluent from the Dowex 50 column (containing neutral and acidic compounds) contained the important component(s). Another separation of shrimp extract on a column of Dowex 50W-X8 was carried out and the water effluent and

2 N-NH_3 eluate were tested individually and in combination (Fig. 4). The results revealed that the potency of the total extract was recovered when acidic and neutral compounds in the water effluent were combined with the amphoteric and weakly basic compounds in the NH_3 eluate. A Potency Probit Analysis showed that there was a significant difference between the stimulatory capacity of the total extract and the NH_3 eluate but that there was no significant difference in the effectiveness of the total extract and the combination of effluent and NH_3 eluate. As in previous, similar separations the water effluent alone did not induce many

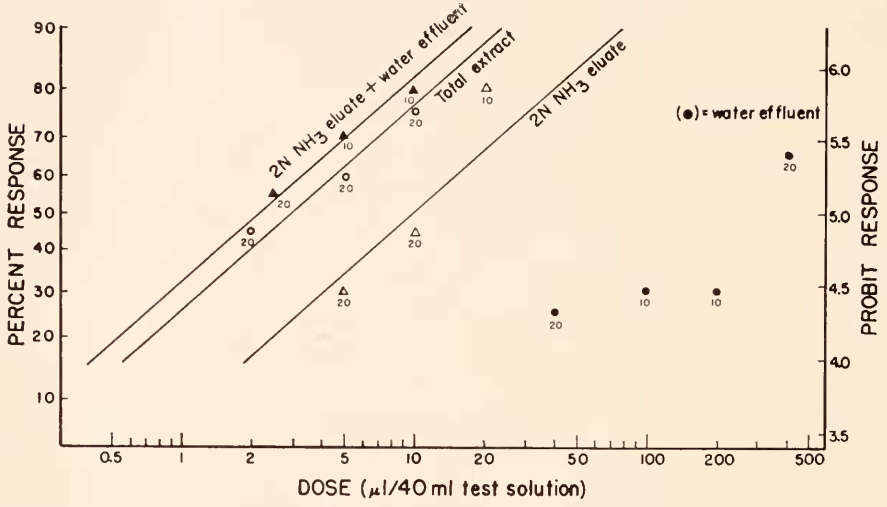

FIGURE 4. Responses of *N. obsoletus* to shrimp extract and to extract components collected in the water effluent and 2 N-NH_3 eluate from a column of Dowex 50W-X8. Linear regression lines were drawn from effective dose values obtained by a Potency Probit Analysis. The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. The following potencies were computed with respect to the potency of the total shrimp extract:

	Upper lim.	0.649		Upper lim.	4.623
2 N-NH_3 eluate	Potency	0.287	2 N-NH_3 eluate + water effluent	Potency	1.389
	Lower lim.	0.069		Lower lim.	0.520

responses at the lower concentrations; however, this material contained a strong response-inducing component(s) which became evident at a concentration of 400 µl./40 ml. (65% response).

Studies of stimulatory components—A summary. The principal response-inducers in shrimp extracts possessed physical properties similar to those of the amino acids and certain other non-volatile, nitrogenous compounds of low molecular weight. These studies also indicated that proteins, labile esters, lipids, and volatile compounds were not important contributors to the response-inducing capacities of the extracts.

Ion exchange separations provided considerable insight into the nature of the principal response-inducers. Neutral compounds collected from a column of mixed bed resin were essentially ineffective even at 100 times the concentration necessary

for the total extract to induce a response of greater than 50%. Separations of extracts on columns of cation exchange resins demonstrated that stimulation was attributable to amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

Effects of small pH changes upon snail response

When necessary, treated (or fractionated) extracts were adjusted to pH 6–8 by a dropwise addition of NaOH. This measure ensured that the pH of each *test* solution was maintained within a considerable narrower range. To determine what

TABLE VI

Responses of N. obsoletus to shrimp extract presented over a narrow range of pH

pH of test solution	Extract concentration tested (μl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
8.0 (unadjusted)	0	10	0	
	2	20	45	
	5	20	60	
	10	20	75	
6.9–7.4**	2	10	50	Upper lim. 3.46
	5	20	60	Potency 0.97
	10	10	70	Lower lim. 0.27
5.8–6.4***	0	10	0	Upper lim. 2.42
	2	20	40	Potency 0.84
	5	20	50	Lower lim. 0.24
	10	20	80	

* Potencies (and confidence limits) were computed with respect to the potency of the extract tested at pH 8.0.

** The pH of the sea water was 6.9 when tests were begun and 7.4 when tests terminated.

*** The pH of the sea water was 5.8 when tests were begun and 6.4 when tests terminated.

effect slight changes in pH had upon snail response, a shrimp extract was tested in sea water which had been acidified by the addition of HCl. The results of these tests are given in Table VI. The snails were equally responsive over the specified pH range (5.8–8.0). In view of these findings that stimulation of the PSR was not effected by slight deviations in pH, test solutions were not buffered. Case (1964) employed electrophysiological techniques to study the sensitivity of crustacean chemoreceptors to amino acids, amines, and related compounds; he reported no change in the stimulatory efficiency of glycine or proline even over a larger pH range. Likewise, Levandowski and Hodgson (1965) found that pH changes in the range 5–8 had no detectable effects upon the responsiveness of lobster chemoreceptors to glutamic acid and certain amines.

Merits of N. obsoletus as a test animal

N. obsoletus proved to be an excellent test animal for studies of chemoreception. This intertidal gastropod is easy to collect and is present in great numbers from the

Gulf of St. Lawrence to the northeast coast of Florida (Abbott, 1954, p. 240). The PSR is a stereotyped response which is convenient for measuring the stimulatory capacities of substances extracted from tissues. With the PSR as the criterion of response, it is possible not only to recognize response-inducing solutions but also to compare in a quantitative manner the response-inducing capacities of closely related solutions. The significance of the latter finding resides in the fact that this snail can be used as a tool for a thorough exploration of the factors (both chemical and physical) which contribute to and influence an integrated response in a marine gastropod. Further, *N. obsoletus* can be tested in small volumes of sea water and is easy to maintain in the laboratory; not a single snail died during the course of this work in which several thousand snails were used.

The development of a convenient bioassay procedure and the use of this procedure to characterize the response-inducing compounds in shrimp extracts have served as the basis for identifying and testing specific compounds, and combinations of compounds, which contribute to the capacity of these extracts to induce the PSR in *N. obsoletus*. The results of the latter experiments will be presented in a separate publication.

Summary

1. The mud snail, *Nassarius obsoletus,* was shown to be an excellent test animal for studies of chemoreception. This snail responds to substances diffusing from dead animals by giving a series of extensions of the proboscis. With this response as the criterion, a bioassay procedure was developed for studying the properties of substances which contribute to the response-inducing capacities of shrimp extracts. *N. obsoletus* can be used in studies in which it is desirable to compare in a quantitative manner the response-inducing capacities of closely related solutions.

2. The principal response-inducing compounds in shrimp extracts were heat-stable, more soluble in polar than in non-polar solvents, non-volatile, of low molecular weight, alkali-stable, partially acid-labile, and resistant to oxidation.

3. Separations of shrimp extracts on ion exchange columns revealed that the principal response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

4. Changes in pH within the range 5.8–8.0 had no detectable effect upon the responsiveness of *N. obsoletus* to a shrimp extract.

LITERATURE CITED

Abbott, R. T., 1954. American Seashells. D. Van Nostrand Co., Inc., New York.

Awapara, J., V. E. Davis and O. Graham, 1960. Chromatographic methods for the identification of biogenic amines. *J. Chromatography,* **3**: 11–19.

Blake, J. W., 1960. Oxygen consumption of bivalve prey and their attractiveness to the gastropod, *Urosalpinx cinerea. Limnol. Oceanog.,* **5**: 273–280.

Brown, A. C., 1961. Chemoreception in the sandy-beach snail, *Bullia. S. A. Journal of Laboratory and Clinical Medicine,* **7**: 160.

Bullock, T. H., 1953. Predator recognition and escape responses of some intertidal gastropods in presence of starfish. *Behaviour,* **5**: 130–140.

Case, J., 1964. Properties of the dactyl chemoreceptors of *Cancer antennarius* Stimpson and *C. productus* Randall. *Biol. Bull.,* **127**: 428–446.

Coe, W. R., 1953. Influences of association, isolation, and nutrition on the sexuality of snails of the genus *Crepidula. J. Exp. Zool.,* **122**: 5–19.

Copeland, M., 1918. The olfactory reactions and organs of the marine snails *Allectrion obsoleta* (Say) and *Busycon canaliculatum* (Linn.). *J. Exp. Zool.*, **25**: 177–227.

Daum, R. J., and C. Givens, 1963. Potency Probit Analysis. U. S. Dept. Agriculture, Biometrical Services. Beltsville, Md.

Dimon, A. C., 1905. The mud snail: *Nassa obsoleta*. *Cold Spring Harbor Monographs No. 5*: 1–48.

Finney, D. J., 1962. Probit Analysis, 2nd ed. Cambridge University Press, Cambridge.

Folch, J., M. Lees and G. H. Sloane Stanley, 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.*, **226**: 497–509.

Frings, H., and C. Frings, 1965. Chemosensory bases of food-finding and feeding in *Aplysia juliana* (Mollusca, Opisthobranchia). *Biol. Bull.*, **128**: 211–217.

Hodgson, E. S., 1955. Problems in invertebrate chemoreception. *Quart. Rev. Biol.*, **30**: 331–347.

Kleerekoper, H., and J. Mogensen, 1963. Role of olfaction in the orientation of *Petromyzon marinus*. 1. Response to a single amine in prey's body odor. *Physiol. Zoöl.*, **36**: 347–360.

Kohn, A. J., 1961. Chemoreception in gastropod molluscs. *Amer. Zool.*, **1**: 291–308.

Levandowski, M., and E. S. Hodgson, 1965. Amino acid and amine receptors in lobsters. *Comp. Biochem. Physiol.*, **16**: 159–161.

Scheltema, R. S., 1961. Metamorphosis of the veliger larvae of *Nassarius obsoletus* in response to bottom sediment. *Biol. Bull.*, **120**: 92–109.

Smith, I., 1960. Chromatographic and Electrophoretic Techniques. Vol. 1. Chromatography. Interscience Publishers, Inc., New York.